Bioactivity evaluation of $TiSiO_4$ nanoparticles in *Lactuca sativa*

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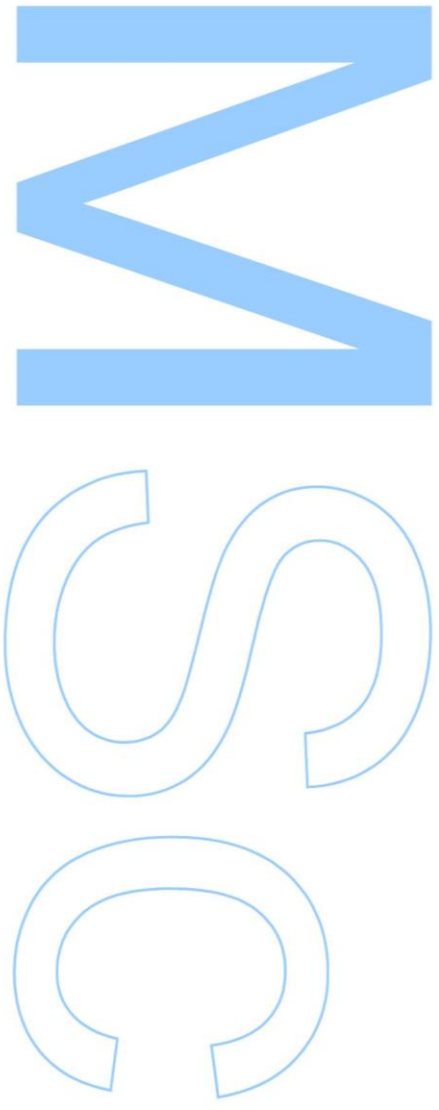

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Bruna Sofia Varzim Ramos

Abstract

Titanium nanoparticles (TiNPs) are increasingly being released in the environment with yet unknown effects, which urges further studies to elucidate their impact on the environment and, particularly, on organisms that may come in contact with these particles. The use of nanoparticles has increased over the years due to their economical relevance and utility in numerous industries, including agri-food technology, medical and pharmaceutical industry and electronics. Several TiNPs formulations are available, of which titanium silicon oxide is one of the least studied. In this work, we aim at clarifying the bioactivity of these particles in plants. For that, we used as model the species *Lactuca sativa*, both an economically important crop and a widely used toxicological model. Seeds were exposed to increasing concentrations (0-100 mg/L) of $TiSiO_4$ and germination rates assessed after 1 week. Then seedlings were exposed to the same concentrations for four weeks in controlled aerated hydroponic conditions. Root and shoot growth rates, oxidative stress (eg., lipid peroxidation, membrane stability) and cytotoxicity were assessed to draw a toxicity profile of these particles in lettuce. Also, the effects of these $TiSiO_4$ nanoparticles in photosynthetic efficiency was assessed by quantifying photosynthetic pigments, fluorescence of photosystem II, starch and total sugars and by measuring gas exchange at leaf level with an infrared gas analyzer (IRGA).

With these data, we unveil the cytotoxic effects of $TiSiO_4$ NPs in *Lactuca sativa*, and the major targets in the cell for this toxicity, and demonstrate that for the doses used no major damages were found, which open perspectives to further studies to confirm that these specific NPs may present some safety level to plants.

Keywords: Lettuce*,* Oxidative stress, Photosynthesis, Titanium dioxide, Toxicity

Sumário

As nanopartículas de titânio (TiNPs) estão cada vez mais espalhadas pelo ambiente e têm efeitos ainda desconhecidos, o que requer estudos adicionais do seu impacto no meio ambiente e, em particular, nos organismos que possam entrar em contato com essas partículas. O uso de nanopartículas aumentou ao longo dos anos devido à sua relevância económica e utilidade em inúmeras indústrias, incluindo tecnologia agro-alimentar, indústria médica e farmacêutica, roupas/calçado e eletrónica. Existem várias formulações de TiNPs, sendo as NPs de dióxido de silício-titânio das menos estudadas. Com este trabalho, pretendemos esclarecer a bioatividade dessas partículas nas plantas. Para isso, utilizamos como modelo *Lactuca sativa*, uma cultura economicamente importante e modelo toxicológico amplamente utilizado. As sementes foram expostas a concentrações crescentes (0-100 mg/L) de $TiSiO_4$ e taxas de germinação avaliadas após 1 semana. Em seguida, foram expostas às mesmas concentrações durante 4 semanas em cultura hidropónica aerodinâmica controlada. Com o objetivo de traçar um perfil de toxicidade dessas partículas em alface, avaliaram-se as taxas de crescimento de raízes/porção aérea, o stress oxidativo (por ex.: peroxidação lipídica, estabilidade da membrana) e citotoxicidade. Além disso, avaliaram-se os efeitos destas nanopartículas de $TiSiO_4$ na eficiência fotossintética quantificando pigmentos fotossintéticos, fluorescência do fotosistema II, amido e açúcares totais e medindo trocas gasosas a nível da folha (IRGA).

Com estes dados, vamos revelar se estas nanopartículas de silício-titânio induzem efeitos citotóxicos em *Lactuca sativa* e os principais “alvos” na célula para essa toxicidade e demonstrar que, para as doses utilizadas, não se encontraram danos significativos, o que abre perspectivas para estudos adicionais para confirmar se estas NPs específicas podem apresentar algum nível de segurança para as plantas.

Palavras-chave: Alface, Stress Oxidativo, Fotossíntese, Dióxido de titânio, Toxicidade

List of abbreviations

APx	ascorbate peroxidase
ATP	adenosine triphosphate
BSA	bovine serum albumin
CMS	cell membrane stability
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
DW	dry weight
EDTA	ethylenediamine tetra acetic acid
eNPs	engineered nanoparticles
FW	fresh weight
GPx	glutathione peroxidase
IRGA	infrared gas analyzer
LHC	light-harvesting complex
MDA	malondialdehyde
MS	Murashige & Skoog
NADPH	nicotinamide adenine dinucleotide phosphate
NPs	nanoparticles
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
PSI	photosystem I
PSII	photosystem II
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
RuBisCO	ribulose biphosphate carboxylase oxygenase
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TiNPs	titanium nanoparticles
TiO_2	titanium dioxide
$TiSiO_4$	titanium silicon oxide
TSS	total soluble sugars
UV	ultraviolet

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1. Introduction

1.1. Nanoparticles

Nanoparticles (NPs) are materials of nanometric size, with variable dimensions from 1 to 100 nm. This reduced size, compared to microscopic materials, leads to differences in NPs properties (González-Melendi et al 2008). These unique physicochemical and electrical properties, along with different materials/techniques used in their coating (for stabilization of NPs), confer them unique advantages to be used in multiple purposes, including cosmetics, electronics, nanomedicine, or in environmental sensors and soil remediation/fertilization (Aitken et al 2006, Buzea et al 2007, Nel et al 2006, Zhang et al 2015).

There are many types of materials that compose NPs, among which carbon and metal elements are the most widely used. Nanoparticles may have natural sources including those NPs derived from eg., volcanic eruptions, photochemical reactions, cosmic dust and dust storms from desert areas (Strambeanu et al 2015). They may also have anthropic origin, which may be occasional (eg., resulting from vehicle gas emissions), or result from engineered NPs (eNPs). Among these, we have the cases of eNPs specifically manufactured for electronic, agro-technological and/or cosmetic/pharmaceutical industries. While natural NPs or those occasionally released from vehicle gas emissions are irregular in size and shape, having no specific coating, the released eNPs generally have more homogeneous dimensions and characteristics (including bioavailability and stability) and generally are coated by compounds such as citrate, polyvinylpyrrolidone (PVP), or polyethylene glycol (PEG) to increase and homogenize their stability properties (Anbarasu et al 2015).

According to their composition and properties, NPs can be divided in several types, such as carbon NPs, metal NPs, zero valence metal NPs, quantum dots, and organic NPs such as liposomes, micelles and dendrimers (Fig 1).

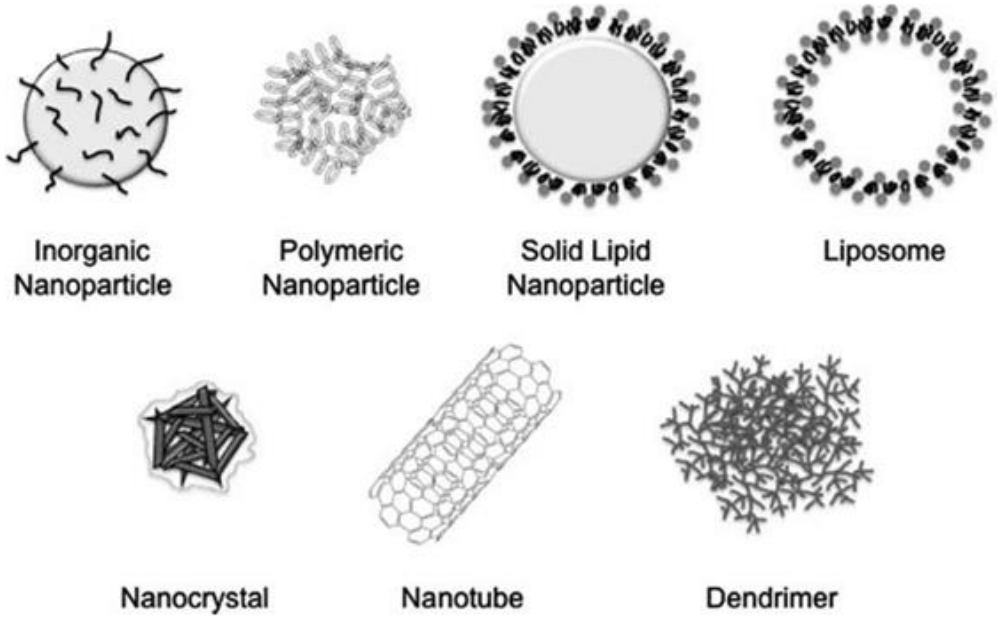


Fig 1 – Types of nanoparticles (adapted from: http://www.pharmatutor.org/articles/review-article-nanoparticle)

1.2. Titanium nanoparticles

Among metal NPs, TiO_2NPs are among the most produced in the world, with an annual production of around 3000 tons per year (Piccinno et al 2012). They are the main constituents of sunscreens, food additives (up to 1-10 µgTi / mg of food), and of industrial paints, being also used in tiles, solar panels and slabs. They also are among the best studied TiNPs for their toxicity in crops (eg, Silva et al 2016). These NPs may have several different denominations based on their structure: anatase, rutile or brookite (orthorhombic) (Fig 2).

Tetragonal forms (rutile and anatase) are the most stable polymorphic forms of TiO_2NPs making them a desirable material for industrial use. TiO_2 are known for having photocatalytic activity and hydrophilic character (they create a closed water film with the capacity to carry pollutants and to clean water) (Ilisz et al 2003).

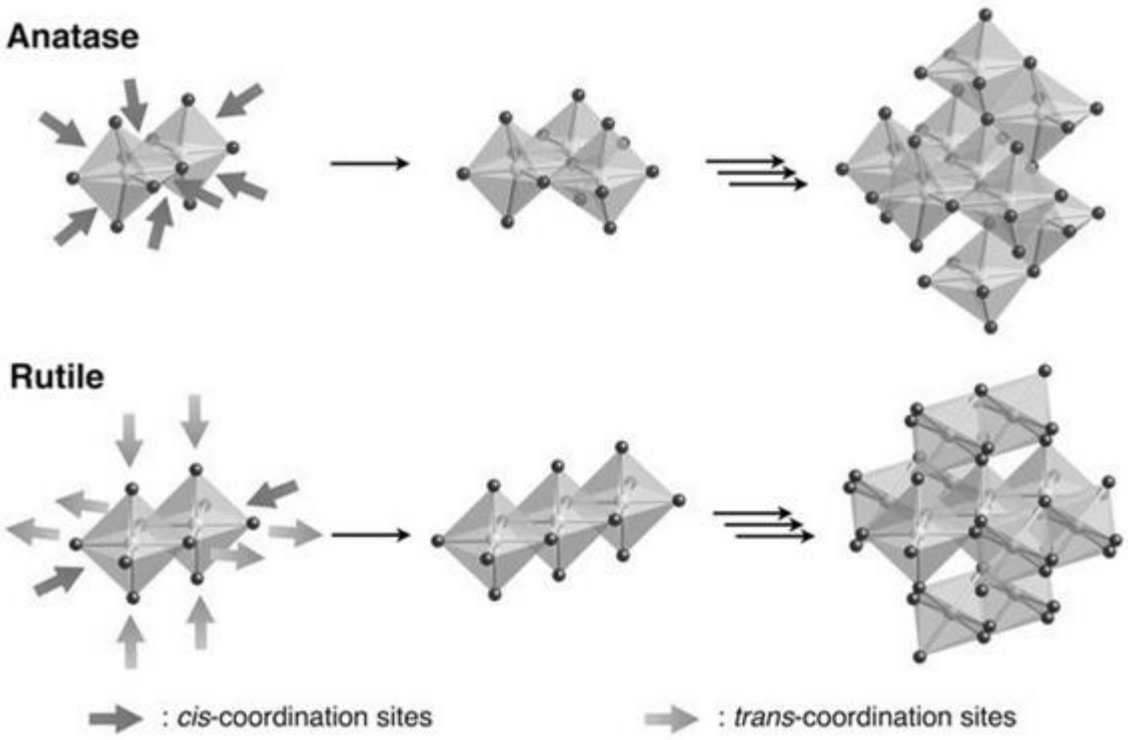


Fig 2 – Crystal structures of the rutile and anatase phases of TiO_2 (adapted from: Satoh et al 2013)

1.3. Titanium nanoparticles toxicity

Studies of geno- and cytotoxicity have been performed on animal cells and less in plants, often showing that damage is dependent on the NP formulation, concentration and coating, among other factors. As example of studies in animals/animal cells, Hussain et al (2010) reported that TiO_2 induced apoptosis, cytotoxicity and ROS (reactive oxygen species) production in *in vitro* cells exposed to high concentrations of TiO_2 NPs.

Hackenberg et al (2011) proved the absence of any DNA damage on human cells of the nasal mucosa and lymphocytes exposed to TiNPs. Other epidemiological studies of TiNPs performed in humans don't demonstrate significant levels of toxicity caused by this type of NPs (Ellis et al 2010; Wild et al 2009).

Due to the focus of this thesis's research being in plants we will detail below the most relevant current state of the art on titanium NPs phytotoxicity.

1.3.1. Titanium nanoparticles toxicity in plants

Studies performed by Song et al (2013) with seeds from *Brassica campestris ssp. napus* var*. nippo-oleifera*, *Lactuca sativa* and *Phaseolus vulgaris* var. *humilis* showed that germination rate was not affected in any concentration used (0, 100, 500, 1000, 2500, and 5000 mg/L). These authors also showed that these NPs were absorbed by

the seed tissues. These results reinforce the idea that TiO_2NPs do not influence seed germination rate, and are supported by other studies on species such as *Triticum aestivum, Oryza sativa* and *Zea mays* (Larue et al 2012). However, there are still contradictory data on maize, with other authors reporting a ~ 50% decrease of germination rate (Castiglione et al 2011). These controversial results may however be justified by the exposure conditions, doses used (the latter authors used higher doses (4.0 %) than the former authors) and biological species/genotypes. Also the type of the TiO_2NPs are relevant, and for example, Larue et al (2012) and Castiglione et al (2011) used TiO_2NPs of lower dimensions than those used by Song et al (2013), which may also affect NPs absorption by the tissues.

The influence of TiO_2NPs on the time needed for germination is also a matter of debate. In general, increased time of exposure to the particles apparently did not affect the germination rates (eg. Song et al 2013). However, when *Triticum aestivum* seeds were germinated in the presence of TiO_2NPs (or larger TiO_2 particles), a stimulatory effect of NPs, especially for 10 mg/L was observed, regarding unexposed seeds. These data support that these NPs may accelerate germination, and even increase seedling's growth (Larue et al 2012).

Similar stimuli were found in spinach seeds (*Spinacea oleracea*) after imbibition in solution containing TiO_2NPs (Zheng et al 2005) and for other species (Clément et al 2013, Tumburu et al 2014). Also in a comparative study, Hatami et al (2014) found that *Salvia mirzayanii*, *Alyssyumhomolocarpum*, *Sinapis alba*, *Carumcopticum* and *Nigella sativa* seeds exposed to TiO_2NPs (up to 80 mg/L) showed a stimulation of their germination rates. A general trend of responses to these NPs remains however complex, as also other species showed to be sensitive to TiO_2NPs, and include *Nicotiana tabacum* (Ghosh et al 2010) and *Mentha piperita* (Samadi et al 2014).

The effects of TiO_2NPs on plant growth have also been studied and, similarly to other NPs, toxicity depends on the sensitivity of the species, NPs size, and exposure conditions (soil vs. hydroponics vs. aerosols, time and dose of exposure). For example hermetic effects have been described in wheat plants exposed to NPs contaminated soil, as increased roots and shoot length and biomass were identified (Rafique et al 2014). On the other hand, toxicity is also reported by other authors. For example, after tomato leaves were sprayed with TiO_2NP (NP uptake was not radicular but mainly stomatal) the growth of the aerial part decreased (Raliya et al 2015).

Subsequent studies showed an accumulation of NPs in fruits and increased lycopene production (Cox et al 2016). Overall, several authors suggest an apparently non-harmful effect of TiO_2NPs particles on root growth / elongation, for example in maize, rice and wheat (Cox et al 2016; Feizi et al 2011; Yang et al 2015). In *Lepidium sativum*

plants growth / elongation was negatively affected by the exposure to NPs (Josko and Oleszczuk 2013). Those authors also showed that light and temperature affect the toxicity of TiO_2NPs. Song et al (2013) showed that *Brassica campestris* and *Phaseolus vulgaris* present root elongation sensitivity to NPs. On other hand, Samadi et al (2014) showed that 100 mg/L was beneficial for root elongation in *Mentha piperita*, and toxicological effects were only observed at 200 mg/L TiO_2NPs. Frazier et al (2013) observed an interesting effect in *N. tabacum* in which the exposure to TiNPs inhibited root growth, but stimulated the appearance of secondary roots. The authors speculated that this effect occurred due to damage in the root apical meristem.

Besides TiO_2NPs, other titanium NPs are found in the market and in the environment, namely titanium silicon oxide NPs. This material is considered to have intermediate properties of silicon dioxide and titanium dioxide. The titanium silicon oxide NPs are mostly used due to their photocatalyst activity in disinfection and sterilization (Kondrakov et al 2014; Cushnie et al 2010) and conversion of carbon dioxide as reported by Tan et al (2006). According to Anpo et al (1986) the titanium-silicon bond enhances the photocatalytic properties of TiO_2.

The effects of TiO_2NPs and SiO_2NPs were also studied simultaneously in some plants revealing promising improvements in drough tolerance (Shallan et al 2016) and some phytotoxic effects on maize seed germination (Karunakaran et al 2016).

Whilst most of the toxicological assays on TiNPs were conducted with TiO_2NPs (anatase and rutile), the studies with titanium silicon oxide are practically unknown, up to the moment restricted to one study in soils with *Avena sativa* (oat), *Zea mays* (corn*), Lactuca sativa* (lettuce) and *S. lycopersicum* (tomato) (Bouguerra et al 2016) and one study with *Vigna radiata* seedlings (Kaur and Maurya 2016). This lack of knowledge on their bioactivity urges the necessity to develop studies on their toxicity in living organisms. As most studies on NPs focus on their toxicity, the uptake and internalization of nanoparticles in plants is also very important.

Studies in *Arabidopsis thaliana* report that nanoconjugates of TiO_2NPs can translocate cell walls and accumulate in specific subcellular locations forming NPs aggregates namely in the vacuole and nuclei (Kurepa et al 2010).

Former studies in pumpkin (*Cucurbita pepo*) have reported the internalization of carbon-coated nanoparticles applied *in planta* by injection and spraying. In both situations, isolated NPs were found in the cytoplasm of epidermic cells near the application point, sometimes forming NPs aggregates. Particle aggregates were also found in adjacent parenchymatic cells and in the cytoplasm of cells close to the vascular core. This result suggests the use of the plants' vascular system for the translocation of NPs in plants' cells (Corredor et al 2009).

1.4. Oxidative stress

1.4.1. General concepts

The oxidative status of a plant is crucial to its survival. Whilst reactive oxygen species (ROS) act as messengers in low doses, under uncontrolled stress conditions the production of ROS may increase, leading to uncontrolled oxidation of several biomolecules including lipids, proteins and nuclei acids.

The main source of ROS production is aerobic respiration. Increased production of ROS activates the antioxidant defenses, eg superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). In healthy plants, ROS are eliminated naturally through SOD, catalase or GPx action as the main enzymatic antioxidant battery and other antioxidant nonenzymatic molecules (Fig 3). Antioxidants are molecules that are able to donate spare electrons to the free radicals thus neutralizing them. This process occurs naturally in every cell to maintain the ROS:antioxidant molecules ratio, preventing cell damage.

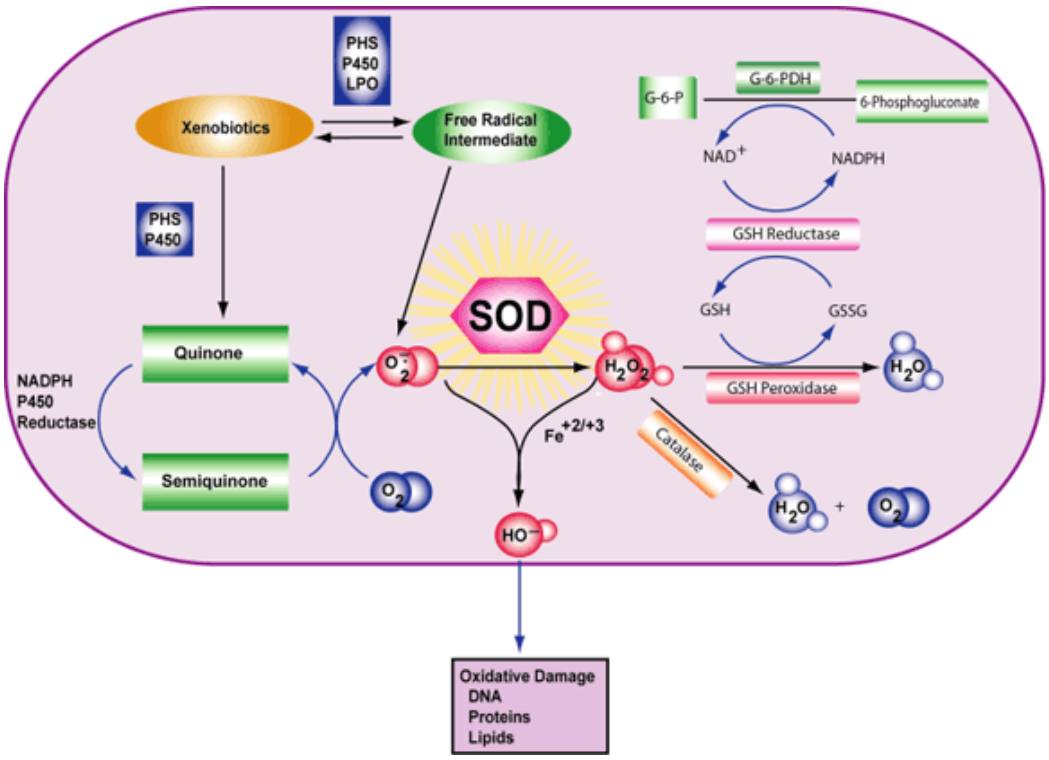


Fig 3 – The role of Reactive Oxygen Species (ROS) in oxidative stress (Source: http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/cell-signaling-enzymes/superoxide-dismutase.html)

1.4.2. Impact of TiNPs on the oxidative status of plants

Although there are already a number of studies on the cytotoxicity and genotoxicity of TiNPs in animal / human cells (Iavicoli et al 2012), there are not enough studies to clearly define if and how TiNPs are phytotoxic and the main metabolic targets of these NPs.

Studies in *Allium cepa* showed that TiO_2NPs have a high potential for interacting with DNA, damaging the root meristem (Demir et al 2014). The same study also showed that smaller size NPs were less toxic than larger ones. In that species, comet analysis also showed that DNA damage increases with TiO_2NPs concentrations. Similar results were observed in *A. cepa* and *N. tabacum*, but with reduction in toxicity at higher concentrations. According to the authors at these concentrations, aggregation and precipitation of the particles occurred, thus preventing their interaction with plants (Ghosh et al 2010). Micronucleus analysis showed that these two species have different susceptibilities in terms of DNA damage, with *N. tabacum* showing more extensive damage at higher NP concentrations than in *A. cepa*. In a study by Klančnik et al (2011) with UV light sources, *A. cepa* did not present genotoxic damage (the authors proposed that ultraviolet radiation led to changes in TiO_2NPs due to their photocatalytic capacity).

Regarding the oxidative stress induced by TiO_2NPs, Pakrashi et al (2014) have shown in root apices of *A. cepa* that, in addition to anaphase disturbances, and other mitotic disorders, the increase in the levels of ROS was dependent on the concentration of TiO_2NPs. The authors attributed the toxicity to this increase in ROS / oxidative stress. This hypothesis of the potential toxicity of TiO_2NPs, due to an indirect effect of ROS increase, was also confirmed for the longer exposure times (4 hours) and for the lower concentrations (12.5 µg/mL).

Another aspect to be taken into account is the different species sensitivities to these NPs. For example, many studies conducted on *A. cepa* show low sensitivity (and few changes in gene expression) between exposed roots and control, even in plants exposed to UV and non-UV light (Koce et al 2014), although DNA damage is detected (Demir et al 2014), and although it does not support other studies that point out that effects on enzymes, such as peroxidase and catalase, were dependent on the concentration of the NPs.

In contrast, in other species, such as *A. thaliana*, significant changes in gene expression were observed in several biological processes (including genes associated with oxidative stress) when seeds were exposed to 500 mg/L. The authors proposed that this is a stress defense mechanism imposed by these NPs (Ze et al 2011).

One of the impacts of the oxidative disorder is the increased production of malondialdehyde (a product resulting from the cascades of lipid peroxidation). One of the targets of this malondialdehyde is membrane phospholipids, leading to cell membrane destruction (assessed by techniques such as the increase of membrane permeability that implies loss of solutes / electrolytes).

Some studies report increased MDA content in plants contaminated with metal ions (eg Ren et al 2011). Aliabadi et al (2016) obtained similar results with TiO_2NPs in wheat, producing high levels of ROS activating the antioxidant battery in the cell, leading to oxidative stress (Mohammadi et al 2013).

1.5. Photosynthesis

1.5.1. General concepts with relevance for NPs interaction

Plants exposed to NPs, by either air or root/soil, may face the translocation of these NPs to the mesophyll, and their interaction with the chloroplasts, ultimately affecting photosynthesis. Therefore, a brief synopsis of the photosynthetic pathways is presented here. Photosynthesis includes two major processes, the photophosphorylation in thylakoidal membranes, and the Calvin cycle in the chloroplast stroma.

Photophosphorylation is a light-dependent reaction as it uses light as an energy source. When light is absorbed by photosystem I, an excited electron enters an electron transport chain to produce ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate).

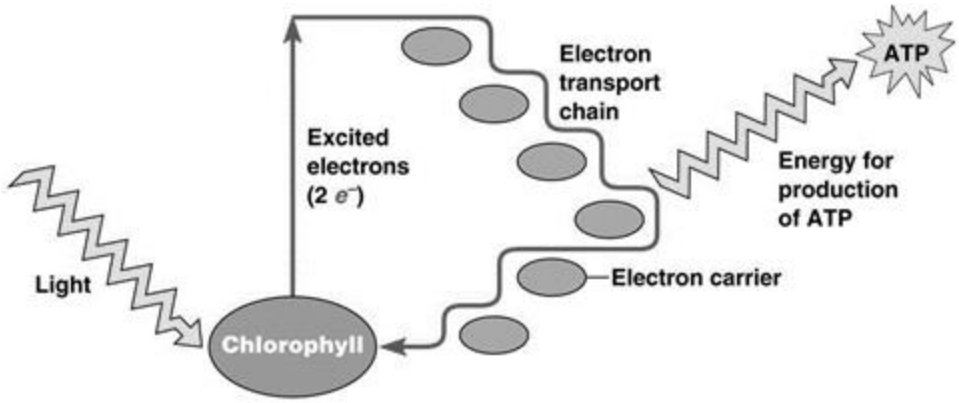


Fig 4 – Schematic representation of photophosphorylation (Source: http://byjus.com/biology/cyclic-photophosphorylation/).

The efficiency of photophosphorylation process may be assessed by the fluorescence of chlorophyll *a* of the PSII (photosystem II), when specific light beams are applied to

dark adapted and/or to light adapted leaves, by specific equipment (eg., fluorimeter) (Fig 4).

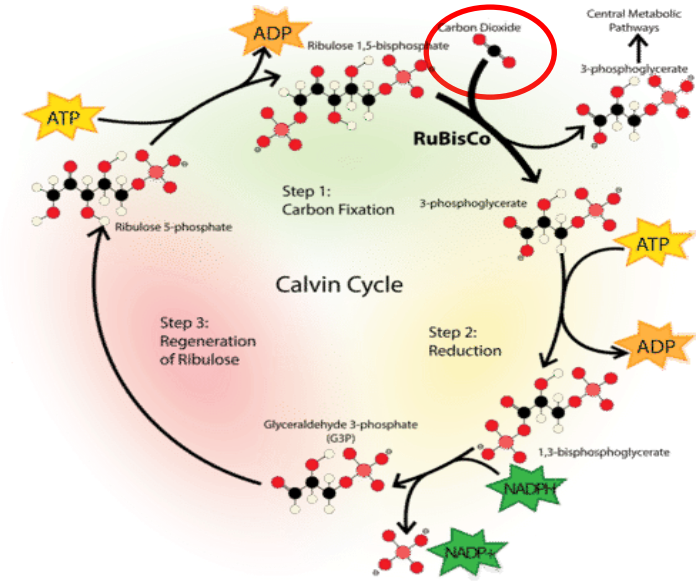


Fig 5 – Schematic representation of the Calvin cycle (Source: https://www.ck12.org/biology/Calvin-Cycle/lesson/Calvin-Cycle-BIO/)

The energy produced previously in the light reaction is used to fix carbon dioxide to carbohydrates in the Calvin cycle. Ribulose bisphosphate carboxylase oxygenase (RuBisCO) captures the CO_2 from the atmosphere and, using the newly formed NADPH, releases three-carbon sugars, which are later combined to form sucrose and starch (Fig 5).

The rate of CO_2 assimilation, transpiration rate, stomatal conductance and intercellular CO_2 concentration can be measured *in situ* on the plants' leaves of with an infrared gas analyzer (IRGA). (Fig 6b)

a) b)

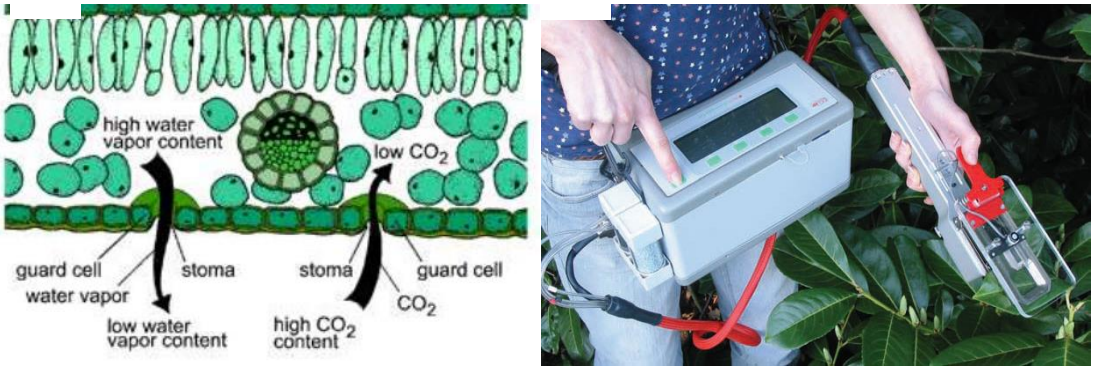


Fig 6 – Gas exchange: a) Schematic representation of gas exchange in plants (Source: http://www.progressivegardens.com/knowledge_tree/stomata.jpg) and b) infrared gas analyzer (IRGA) (source: http://www.concordscientificdevices.com/plant-science/lcisd.html)

1.5.2. Impact of TiNPs on the photosynthesis

The impact of titanium NPs on the photosynthetic capacity of plants remains a crucial topic of discussion, and these studies are restricted to the photosystem of some algae, such as *Chlorella* (Comotto et al 2014). After exposure to TiO_2NPs*, A. thaliana* chloroplasts increased the expression of genes associated with LHC (light-harvesting complex) of photosystem II (LCHII), as well as increased formation of thylakoidal membranes, increasing the absorption of light in the red and blue areas. The authors suggested a very positive effect of these NPs on the stimulation of photosynthesis by LHC stimulation, electron transfer to photosystem I (PSI), and even for oxygen release (Ze et al 2011).

In contrast, Larue et al (2012), working with *Triticum aestivum*, showed not only an effect of the size of the TiO_2NPs in their absorption and accumulation, but also that the species was not sensitive (for the doses tested) at the level of germination, respiration and photosynthetic, nor suffered oxidative stress. However, there have been no statistical correlations between growth and photosynthesis data in plants exposed to these NPs. Gao et al (2013) sprayed *Ulmus elongata* seedlings with extremely high doses of TiNPs (0.4 % ~ 4 g/L) and showed decreases in the photosynthetic rate.

Using radioactive carbon labeling, the authors claim to find no effect on stomatal opening and, on the other hand, while detecting decreases in nitrogen, found no significant changes in other photosynthetic nutrients. Fourier spectroscopy showed changes in carbohydrate and lipid metabolism, which were associated with the potential toxic effect of TiO_2NPs. Thus, the impact of NPs on the synthesis of metabolites like sugars, starch, and other compounds associated with carbon metabolism requires more studies.

Objectives

The hypothesis in this project is that the effects of $TiSiO_4$ on plants is dependent on the concentration, and involves changes in the oxidative status and in the photosynthesis.

For example, we hypothesize that exposing seeds /seedlings to low doses (eg., 1 mg/L) of $TiSiO_4$ may not impair plants' physiology and growth, but higher doses (eg., 100 mg/L) will have toxicological effects, with increased oxidative stress and cytotoxicity, and impaired photosynthesis.

To address this hypothesis the main objectives of this project are:

a) to assess the impact of $TiSiO_4$ on germination and growth rates of the model species lettuce;

b) to unveil if and how major biochemical/metabolic pathways are disturbed by these NPs, namely those related with oxidative stress, and those related with photosynthesis;

c) to identify best (most sensitive) endpoints that may be used in assessing these NPs (and eventually others) toxicity in plants.

As a model species, we will use lettuce (*Lactuca sativa*), a widely used crop also used as toxicological model growing under controlled conditions as recommended by OECD (1984).

2. Methods

2.1. Exposure conditions and growth assays

2.1.1. Nanoparticles solutions

$TiSiO_4$ nanoparticles were purchased from Sigma Aldrich (St. Louis, MO USA), with a purity of 99.8 %. According to the manufacturer, the particle size is <50 nm in the form of nanopowder.

From the commercial powder, stock suspensions (1 g/L) were prepared in sterile water and sonicated for 20 min. The pH was set to 5.7. The stock suspension is used to prepare the final concentrations: 1, 10, 50 and 100 mg/L, by adding the appropriate volume of NP stock and 1/10F MS medium (Murashige & Skoog 1962) (pH 5.7). The control group was irrigated with 1/10F MS medium only.

2.1.2. Plant material and exposure

Lactuca sativa var. *Maravilha das 4 Estações* seeds were germinated on Petri dishes for 1 week containing the different concentrations of NPs (50 seeds/dish). Germination took place in controlled conditions: 16h /8h (day/night) photoperiod, low light intensity, relative humidity of ~ 40 %, 22±4 °C temperature.

After 1 week, germination rates were determined. Seedlings were then transferred to hydroponic culture (20 plants/box with volume of aerated irrigation solution of 700 mL). The solutions were kept in bubbling to aerate the root system and prevent the sedimentation of the NPs. These controlled conditions are essential to ensure that only the NPs concentrations are the changing variable.

Oxidative stress parameters and chlorophyll fluorescence were assessed with fresh plant material. *L. sativa* was then stored at -20 ºC for the following protocols.

2.1.3. Plant growth assessment

After 4 weeks, seedling's growth (root and shoot) and fresh weight were assessed. Root and shoot fresh and dry weights and length were measured. These results give a general information of the effects on biomass. Growth and morphological aspects (eg., chlorosis, nanism, necrosis) were essential to assess the impact on relevant phenotypic/yield parameters in this crop.

2.2. Extraction and quantification of soluble proteins

From exposed plants (~ 4 weeks), 0.5 g of leaf and root were weighed and samples were macerated (on ice) with 5 mL of 0.2 M phosphate buffer (pH 7.5), containing 0.5

mM Na_2 EDTA, 1 mM PMSF, 0.2% Triton X-100 (v/v), and 2 mM dithiothreitol. The extract was collected into falcon tubes and centrifuged at 4 °C for 30 min at 6000 g (Heareus, USA).

Quantification of total soluble proteins was obtained by the Bradford method (1976) according to manufacturer's instructions (Sigma, USA). Briefly, 25 µL of extract was placed in 1.5 mL cuvettes, to which we added 750 µL of Bradford at room temperature. The mixture was inverted and stored at room temperature for ~10 minutes. Absorbance was read at the wavelength of 595 nm (Thermo Fisher Scientific spectrophotometer, Genesys 10-uvS).

Protein concentration per gram of fresh tissue was determined by the equation y = (Abs 595 nm - 0.0128) / 0.117 obtained from a standard line of concentrations of bovine serum albumin (BSA, Merck, DE).

2.3. Lipid peroxidation by quantification of malondialdehyde (MDA)

Determination of lipid peroxidation by the quantification of malondialdehyde (MDA) was obtained by homogenizing 100 mg of leaf tissue in 1.5 mL of 0.1% trichloroacetic acid (TCA) solution. The extract was transferred to 2 mL microtubes. It was vortexed for 30 seconds and then centrifuged for 10 min at 10000 g. Supernatant (500 µL) was collected and in two 2 mL microtubes were pipetted respectively:

A) 250 µL of the supernatant into a 2-mL tube and 1 mL 20% TCA + 0.5% TBA (thiobarbituric acid) was added to the positive control.

B) 250 µL of the supernatant to another 2-mL microtube and then 1 mL 20% TCA was added for negative control.

The tubes were placed in thermoblock at 95 °C for 30 min and after that time were cooled immediately on ice (≈ 10 min). They were then centrifuged for 10 min at 10000 g and the absorbance of the supernatants at 600 nm and 532 nm using the blanks (20% TCA negative and 20% TCA + 0.5% TBA positive) was read. The calculation of the MDA equivalent is given by the equation: $A= [(Abs\ 532^{+TBA}) - (Abs\ 600^{+TBA}) - (Abs\ 532^{-TBA} - Abs\ 600^{-TBA})]$, where +TBA indicates the positive control and -TBA indicates the negative control.

The concentration of MDA equivalent (nmol/mL) was calculated using the formula: (A / 157.000) x 10^6. Finally, the MDA concentration per gram of fresh weight (FW) was calculated, taking into account the extraction volumes used: [MDA] / mg FW = (MDA equivalent x $V_{extraction}$) / sample weight (mol MDA equivalent / mg FW).

2.4. Cell membrane stability (CMS)

Membrane integrity was determined by the cell membrane stability (CMS) method. Leaves of approximately similar dimensions (≈25 mg) were collected and weighed (FW0). They were incubated in 50 mL falcon tubes with 10 mL sterile water and held at 25 °C for 24 hours.

The electrical conductivity (µS) of the extract (L1) was measured using a conductivity meter (Consort, USA). The samples were autoclaved for 1 hour, measuring the conductivity of the sealed tubes (L2). The percentage of membrane damage (MD) is estimated by the equation: % MD = (L1 / L2) x 100.

2.5. Photosynthesis

2.5.1. Gas exchange and chlorophyll fluorescence

The rate of CO_2 assimilation, transpiration rate, stomatal conductance and intercellular CO_2 concentration were measured *in situ* on leaves of *Lactuca sativa* with an infrared gas analyzer (IRGA, LCpro +, ADC, Hoddesdon, UK) according to Dias et al (2013).

Chlorophyll fluorescence was determined with a portable fluorimeter (FMS 2, Hansatech Instruments, Norfolk, England). The leaves were adapted to the dark for 30 minutes. After this time, the minimum fluorescence (F0) was measured and immediately after a flash of intense light (> 1500 µmol/m^2 s), the maximum fluorescence (Fm) was recorded. The leaves were again exposed to normal conditions and adapted to light. After 30 minutes, the minimum fluorescence (F0 ') was measured and immediately after a flash of light (> 5000 µmol/m^2 s), the maximum fluorescence (Fm') was recorded. The following parameters were calculated:

Fv/Fm = (Fm – Fo)/Fm (maximum efficiency of PSII when reaction centers are closed).

The values of basal fluorescence (F0), maximum fluorescence (Fm), variable fluorescence (Fv = Fm-F0), the Fv / Fm ratio obtained in dark-adapted leaves were analyzed according to Maxwell and Johnson (2000).

2.5.2. Photosynthetic pigments content

Photosynthetic pigments content was calculated according to Sims and Gamon (2002). To summarize, 100 mg of leaves were macerated with 1.5 mL of acetone: Tris 50 mM (80:20) buffer (pH 7.8), vortexed for 30 sec and centrifuged for 10 min at 10000 g and at 4 °C. The supernatant was transferred to a falcon covered with aluminum foil, thereby keeping the sample under dark conditions. Buffer (1.5 mL) was added to the residue, and centrifuged again for 10 min at 10000 g and at 4 °C. The supernatant was added to the falcon tube which was in the dark. Absorbance reading of the supernatant

was performed at 663 nm, 537 nm, 647 nm and 470 nm on a Thermo Fisher Scientific (Genesys 10-uvS) spectrophotometer.

The blank only contained the extraction buffer. The pigment contents were calculated based on the following equations:

Chlorophyll *a* = 0.01373 A663 – 0.000897 A537 – 0.003046 A647;

Chlorophyll *b* = 0.02405 A647 – 0.004305 A537 – 0.005507 A663;

Carotenoids = (A470 - (17.1 x (Chl *a* + Chl *b*) – 9.479 x Anthocyanins)) /119.26;

Anthocyanins = 0.08173 A537 – 0.00697 A647 -0.002228 A663.

2.6. Total sugars (TSS)

The concentration of TSA was determined according to Irigoyen et al (1992). Frozen leaves (~50 mg) were macerated with 10 mL of 80% ethanol. The homogenate was placed in a water bath at 80 °C for 1 hour and then placed on ice for 10 min.

The samples were vortexed and then centrifuged for 10 min at 10000 g at 4 °C. Supernatant (30 µL) was removed and 0.75 mL of an anthrone solution (40 mg anthrone in 20 mL H_2SO_4) was added. The resulting solution was placed at 100 °C for 10 min, and then 15 min on ice. Absorbance of the supernatant was read at 625 nm (Thermo Fisher Scientific spectrophotometer, Genesys 10-uvS). The blank consisted of a solution of 0.75 mL of anthrone and ethanol. The TSA concentration was determined from the standard glucose curve, $y = 0.0283x – 0.0025$. ($R^2 = 0.9978$).

2.7. Starch

The starch extraction was performed according to Osaki et al (1991).

To the residue resulting from extraction of TSS, 5 mL of 30 % perchloric acid was added. The homogenate was placed in a water bath at 60 °C for 1 hour and then cooled on ice for 10 min.

The samples were vortexed and then centrifuged for 10 min at 10000 g, 4 °C. Supernatant (30 µL) was removed and 0.75 mL of anthrone solution was added. The resulting solution was placed at 100 °C for 10 min and then placed on ice for 15 min to stop the reaction. Absorbance reading of the supernatant was performed at 625 nm (Thermo Fisher Scientific spectrophotometer, Genesys 10-uvS). For the blank, 0.75 mL of anthrone was used with 30 µL of perchloric acid.

The starch concentration was determined from the glucose standard curve $y = 0.051x – 0.002$. ($R^2 = 0.9965$).

2.8. Statistical analysis

In order to test the effect of the increasing concentrations of titanium silicon oxide nanoparticles in the different parameters, GraphPad Prism 7 software (https://www.graphpad.com/scientific-software/prism/) analysis was performed.

Data from the plants' germination, growth, oxidative stress and photosynthesis were analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test to find the significant differences between the control and the tested concentrations of NPs (for $p<0.05$).

3. Results

3.1. Germination and Plant Growth assays

The seeds presented high germination rates in every tested concentration of the NPs. According to Fig 7, the germination rate of *Lactuca sativa* cv. *Maravilha das 4 Estações* was not significantly affected by the $TiSiO_4$ NPs. The plants also presented similar appearance, with no necrotic spotting or leaf chlorosis. (Fig 8b). According to the statistical analysis, no significant differences were found ($p<0.05$).

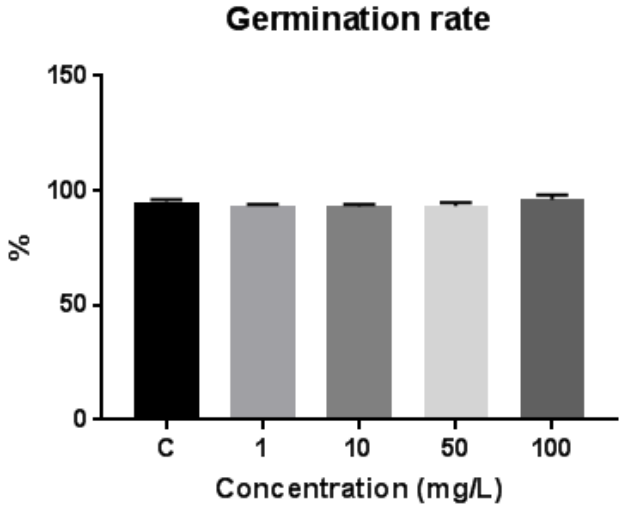


Fig 7 – Germination rates of *L. sativa* exposed to $TiSiO_4$ NPs.

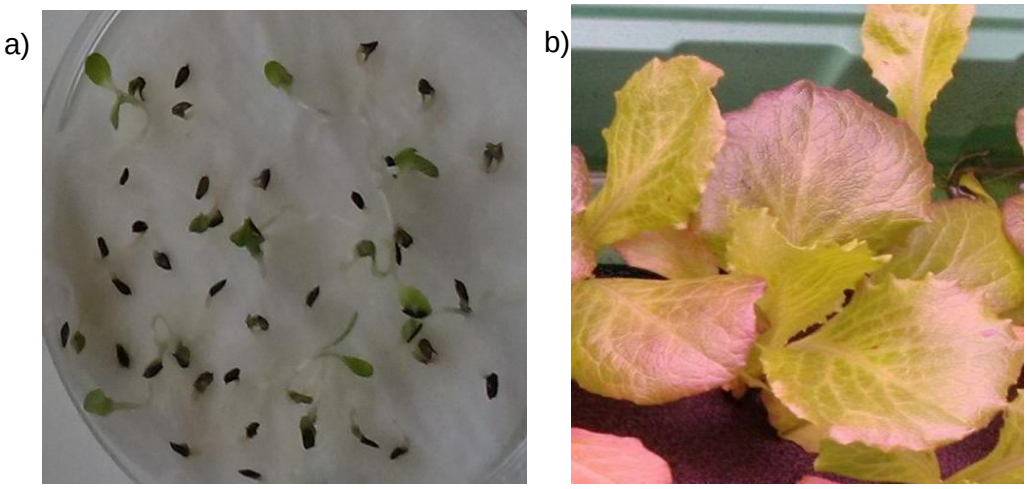


Fig 8 – Photographs of *L. sativa* : a) seedlings 5 days after germination; b) plants after 3 weeks of hydroponic culture.

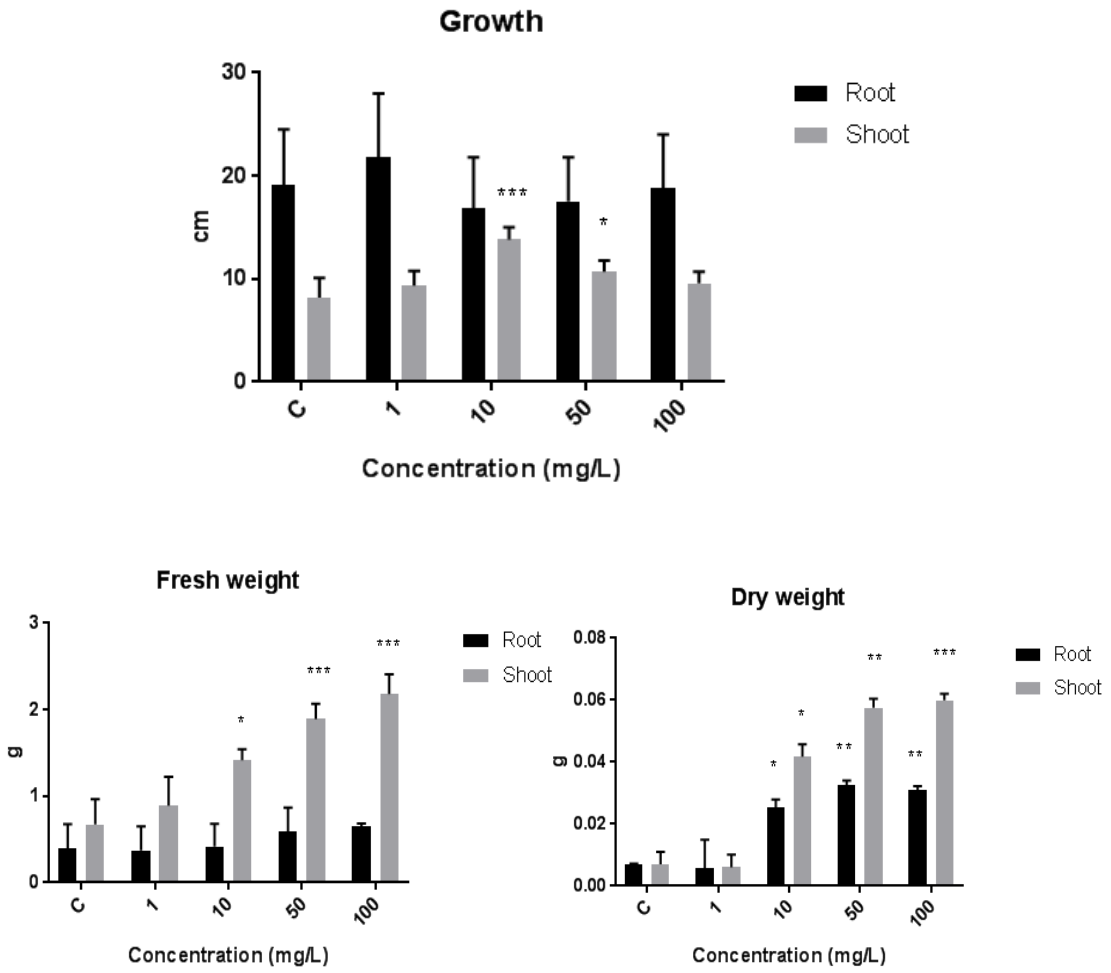


Fig 9 – *L. sativa* growth: a) root and shoot length; b) fresh weight, c) dry weight. Asterisks indicate significant differences for: *p<0.05, ** p<0.01, *** p<0.001

Regarding the effect of the $TiSiO_4$NPs on plants' growth, we verified minimal effects of these NPs relatively to the control (Fig 9a). Overall, the plants showed an increased aerial portion for the higher concentrations of NPs exposure (significant for the 10 and 50 mg/L doses).

The weight of the samples also showed an increase in the higher concentrations of NPs, both in fresh and dry weight having significant results for the 10, 50 and 100 mg/L doses in the aerial portion (regarding the fresh weight); and both root and shoot for the dry weight results (Fig 9b,c).

3.2. Cell membrane stability (CMS)

The most affected plants in terms of cell membrane damage in *L. sativa* were the ones exposed to 50 and 100 mg/L NPs (Fig 10). However, no significant changes were detected for any concentration.

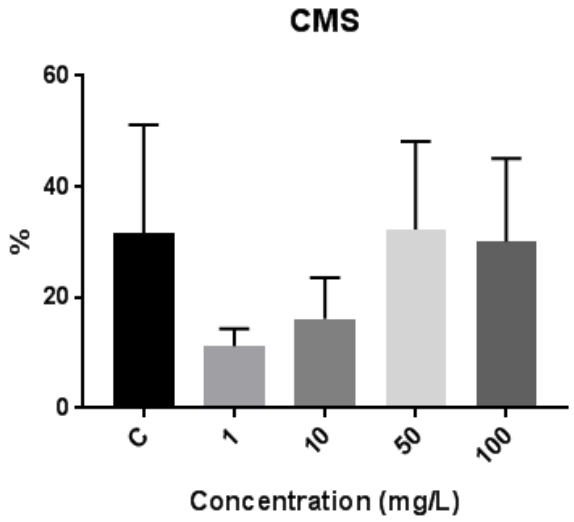


Fig 10 – Cell membrane permeability (%) in *L. sativa*.

3.3. Quantification of malondialdehyde (MDA)

The accumulation of malondiadehyde (MDA) as a result of lipid peroxidation was analyzed in association with oxidative stress damage. The results showed a decrease of MDA levels in the plants exposed to NPs (1, 10, 50 and 100 mg/L) relatively to the control. However, this trend to decrease was not significant except for the 50 mg/L concentration for ($p<0.05$) (Fig 11).

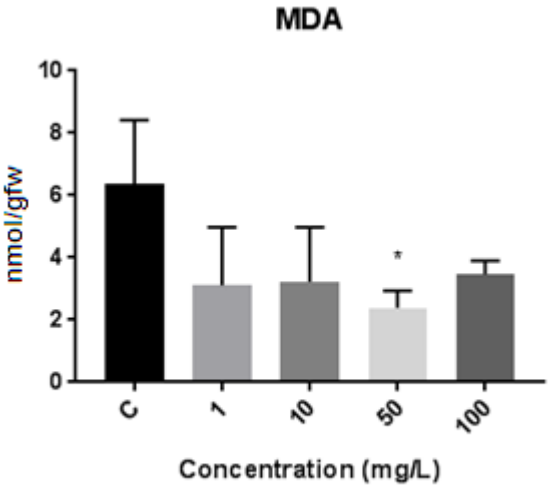


Fig 11 – Accumulation of malondialdehyde (MDA) in *L. sativa*. Asterisks indicate significant differences for: *p<0.05

3.4. Pigment content

The chlorophyll *a* content was similar in all concentrations of $TiSiO_4$ NPs exposure and the control, except for the 50 mg/L concentration which presented higher content (Fig 12a).

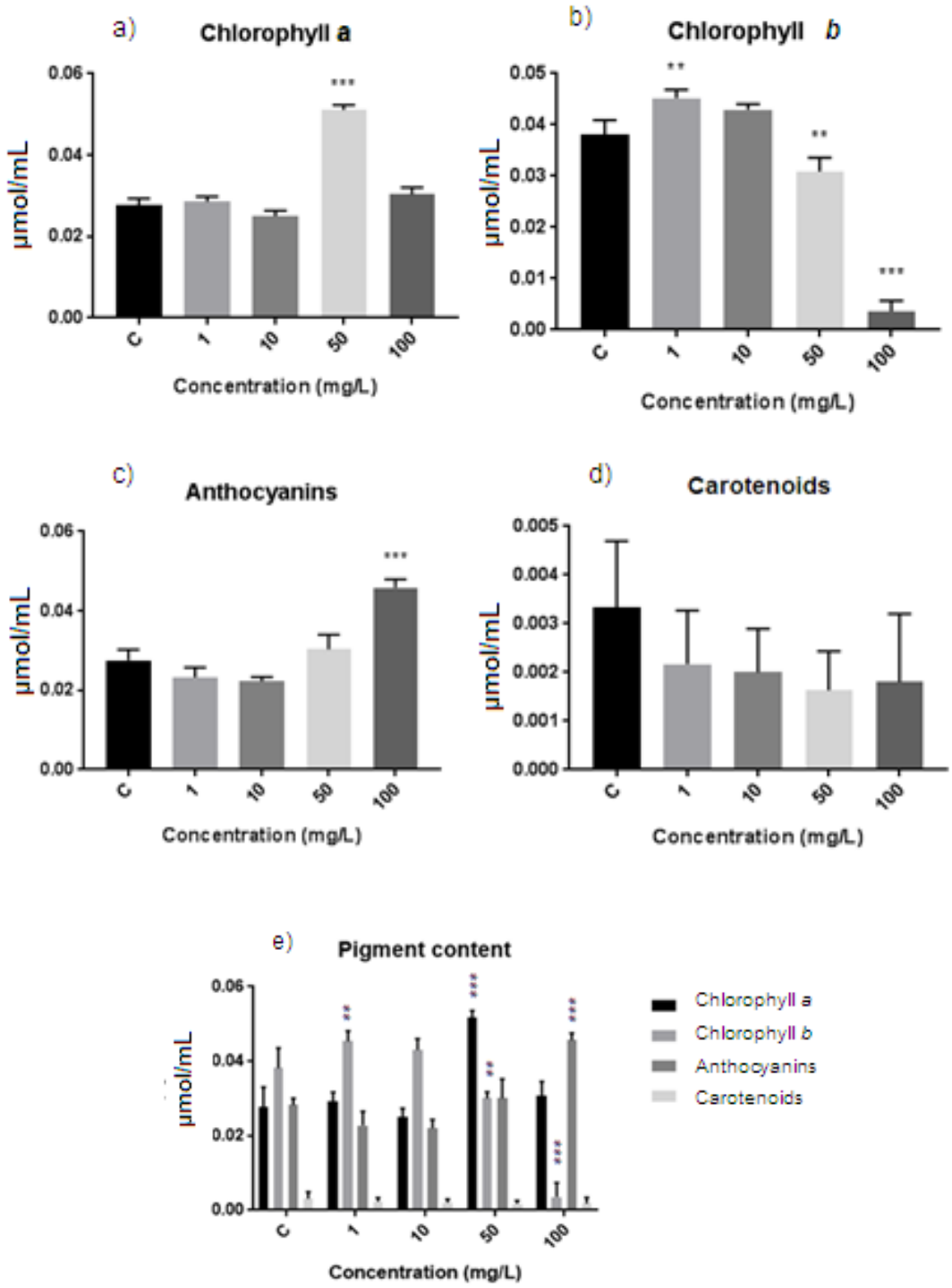


Fig 12 – Content of: a) chlorophyll *a*; b) chlorophyll *b*; c) anthocyanins; d) carotenoids; e) overview of photosynthetic pigments content in *L. sativa*. Asterisks indicate significant differences for: *p<0.05, ** p<0.01, *** p<0.001

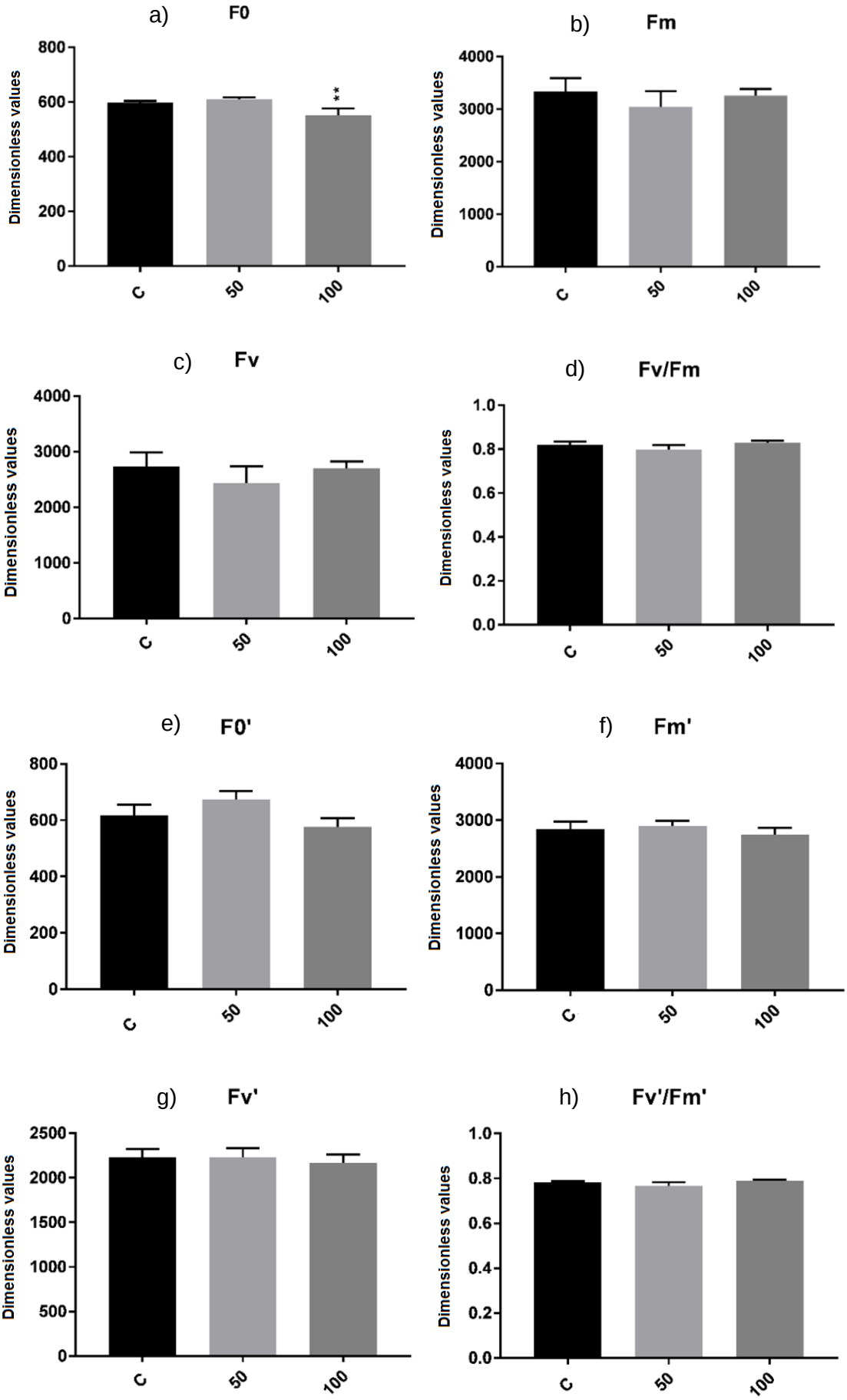
The chlorophyll *b* content increased in the 1 and 10 mg/L concentrations and then decreased in the higher NPs concentrations (50 and 100 mg/L). The results obtained for the 1, 50 and 100 mg/L concentrations presented significant changes (Fig 12b).
Anthocyanins presented a decrease in lower concentrations of $TiSiO_4$ NPs (1 and 10 mg/L) and increased in higher concentrations (50 and 100 mg/L). The 100 mg/L concentration presented higher anthocyanins content than any other tested NPs doses (Fig 12c) and this result has statistical significance.
Carotenoids levels decreased as the $TiSiO_4$ NPs concentration increased (Fig 12d). However, no significant differences were detected.

Overall, the carotenoids content, relatively to the other photosynthetic pigments was lower for all tested concentrations of the $TiSiO_4$ NPs. Both chlorophyll *a* and *b* present the highest contents in the plants' cells (Fig 12e).

3.5. Photosynthesis

Observing the fluorescence results of photosystem II (Fig 13), in dark-adapted plants, the basal fluorescence (F0) had, compared to the control, significantly lower levels in the higher concentration (100 mg/L). For the maximum fluorescence (Fm), all concentrations presented high levels not having any significant differences. At the variable fluorescence (Fv), the 50 mg/L presented a difference relatively to the control (non-significant). The 100 mg/L concentration presented similar results relatively to the control.
The Fv/Fm ratio was similar to all concentrations, indicating that the plants were healthy (the results were non-significant).
In light-adapted plants, a slight increase in F0' was observed, the 50 mg/L concentration having higher value of basal fluorescence. The maximum fluorescence (Fm') had lower values in light-adapted plants comparing to dark-adapted plants. The same results were found for the variable fluorescence (Fv'). The Fv/Fm ratio demonstrates that the dark-adapted plants were healthy (ratio of ~0,8).

Fig 13 (next page) – Results of basal fluorescence, maximum fluorescence, variable fluorescence and health status in dark-adapted plants (a-d) and light-adapted (e-h). Asterisks indicate significant differences for: ** $p<0.01$


a)
F0
Dimensionless values
800
600
400
200
0
**
C
50
100
b)
Fm
Dimensionless values
4000
3000
2000
1000
0
C
50
100
c)
Fv
Dimensionless values
4000
3000
2000
1000
0
C
50
100
d)
Fv/Fm
Dimensionless values
1.0
0.8
0.6
0.4
0.2
0.0
C
50
100
e)
F0'
Dimensionless values
800
600
400
200
0
C
50
100
f)
Fm'
Dimensionless values
4000
3000
2000
1000
0
C
50
100
g)
Fv'
Dimensionless values
2500
2000
1500
1000
500
0
C
50
100
h)
Fv'/Fm'
Dimensionless values
1.0
0.8
0.6
0.4
0.2
0.0
C
50
100

Gas exchange

For the photosynthetic rate parameters (Fig 14a), the 1 and 10 mg/L concentrations presented higher values than the control (1 mg/L presented significant changes and the 10 mg/L concentration non-significant). These results showed that NPs positively affected the photosynthetic rate. Similar results were obtained for the transpiration rates in exposed plants (Fig 14b), having a slight trend to decrease in the 10 mg/L concentration (non-significant).

The 1 mg/L concentration induced the highest value in stomatal conductance (Fig 14c), while the 10 mg/L dose presented similar results relatively to the control although non-significant changes were detected (Fig 14c). For the intercellular CO_2 concentration in plants, the control showed higher levels of CO_2 than the exposed plants (1 and 10 mg/L have significant changes) as shown in the fig 14d below.

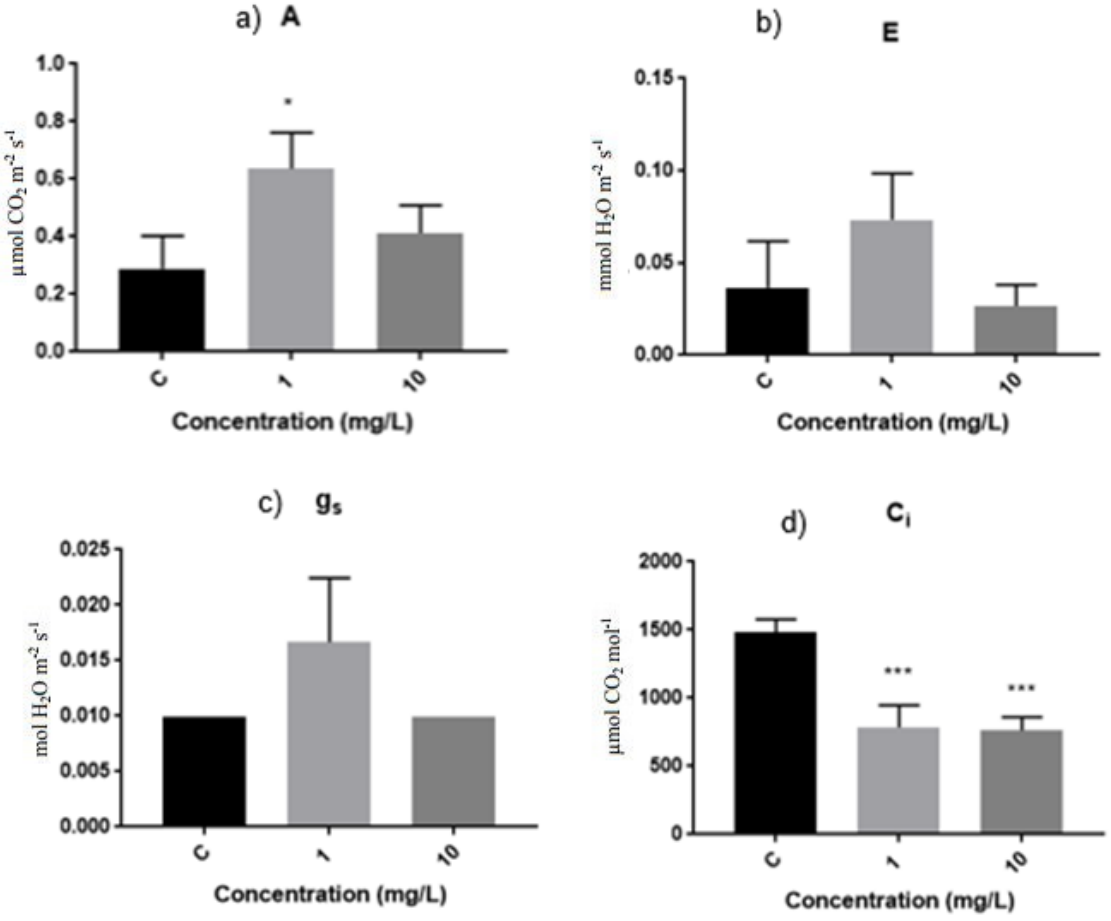


Fig 14 – Results of: a) Photosynthetic rate, b) Transpiration rate, c) Stomatal conductance to H_2O, d) Intercellular CO_2 concentration in *L. sativa.*

Asterisks indicate significant differences for: *p<0.05, ** p<0.01, *** p<0.001

3.6. TSS, starch and soluble proteins

The total sugars presented higher values as the concentration of NPs increased, except for the 50 mg/L that showed a decrease (only 1 mg/L had significant changes) (Fig 15a).

Overall, the starch content presented lower values in the higher concentrations of NPs (10 and 100 mg/L) except for the 50 mg/L concentration where higher contents were observed (similar values to the control and the 1 mg/L). Despite this trend of changes, no significant changes were verified (Fig 15b).

The soluble proteins content showed a hormetic profile with the increase of the NPs concentration. The soluble proteins content increased in the 1 mg/L concentration and then for higher NP concentrations, the content of soluble proteins decreased (Fig 15c). The results obtained for the concentrations of 1 and 100 mg/L were significant.

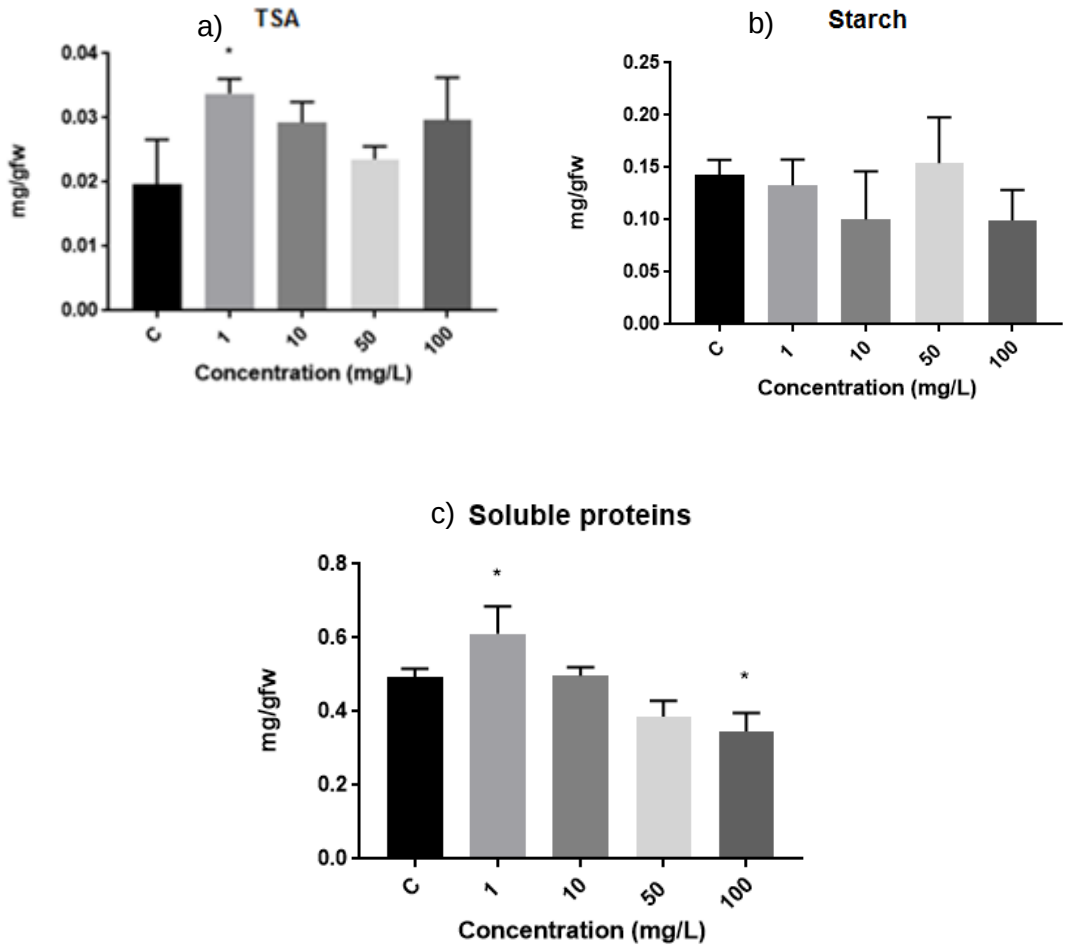


Fig 15 – Levels of: a) total soluble sugars (AST); b) starch; c) soluble proteins in *L. sativa*. Asterisks indicate significant differences for: *p<0.05

4. Discussion

This thesis contributes to better assess the bioactivity/toxicity of $TiSiO_4$ in crops. The obtained results showed no negative effects on the plants' germination rates. The same result was reported by other studies of TiO_2 NPs in different species such as *S. lycopersicum* (Song et al 2013), *T. aestivum* (Larue et al 2012) and *Zea mays* (Castiglione et al 2011).

Ghosh et al (2010) showed inhibition of root elongation in plants when exposed to TiO_2 NPs (germination did not occur under TiO_2 exposure). However, Clement el al (2013) reported a stimulation of plant growth by nanoparticles in *Linum usitatissimum*. In *L. sativa* also showed an increased aerial portion in higher concentrations of NPs exposure. The root and shoot length was increased in *L. sativa* in the higher concentrations of NPs.

The plants' biomass increased significantly relatively to the control, especially in the higher concentrations (50 and 100 mg/L) both in fresh and dry weight. One hypothesis raised by Larue et al (2012) is that oxidative stress generated by TiNPs could render the cell wall more permeable and increase water flow. Previous studies suggest that the increase in weight may be due to increased water uptake and / or root thickness (Silva et al 2016).

Plants that are grown in stressful environments (eg. drought, NPs exposure) tend to accumulate free radicals. Peroxidation of cell membrane lipids is an indicator for toxic substances present in the plants' cells and MDA is one of the constituents formed in the process of detoxification (Shaw & Hossain, 2013). The higher concentrations (50 and 100 mg/L) of NPs exposure showed higher percentage of membrane damage than the lower concentrations. Some authors hypothesize that the increase in ROS production causes the DNA damage in cells exposed to TiNPs (Guichard et al 2012). Studies in *A. cepa* (Ghosh et al 2010) have shown an increase in lipid peroxidation that is related to oxidative stress. The results indicated oxidation influence on cell membrane integrity. However, this interaction is still poorly studied in plants (Silva et al 2016). Our results show a decrease in the MDA levels in the higher concentrations of the NPs, though non-significant relatively to the control that may indicate that there was no increase in the lipid peroxidation. However, there are other products that may be formed during lipid peroxidation, as it is only one of the markers of oxidative stress. There are several other complementary methods for detecting the presence of ROS, or related enzymes, as a mechanism of plants' defense against environmental stresses. These other methods combined could provide a more complete overview.

The oxidative stress caused by the NPs in terms of MDA levels showed significant differences in the 50 mg/L dose. Studies in *Cucumis sativus L*. reported oxidative stress caused by the exposure to TiNPs (Cox et al 2016). The plants showed an increased catalase activity and decreased activity of ascorbate peroxidase (APx). Geno and cytotoxicity caused by oxidative stress was also reported in *Phaseolus mungo* and *Sorghum vulgare* studies performed by Jadhav et al (2011).

The light absorbed by chlorophyll molecules can be used in the process of photosynthesis, dissipated as heat, or re-emitted as chlorophyll fluorescence (Maxwell and Johnson 2000). The chlorophyll fluorescence analysis is based on electron transport chain analysis. The light absorbance by chlorophyll molecules (detected by fluorescence measuring devices – eg. fluorimeter), and the efficiency of photosystem II are strongly related. Results of energy dissipation and photochemical efficiency can be obtained by measurements of chlorophyll fluorescence. After a dark period, when the light intensity is enough to generate a stimulus on a leaf, there is a transient increase in chlorophyll fluorescence which is the result of electron reduction by thylakoid membrane transporters (Murchie et al 2007). In dark-adapted leaves, a very low intensity light is switched on to induce the transport of electrons through the PSII (high enough to create a minimum value of chlorophyll fluorescence – Fo). Measurement of Fo and its light-adapted equivalent Fo' is critical for fluorescence analysis (Peltier and Cournac 2002). Kummerova et al (2006) suggest that hydrocarbon phytotoxicity affected the primary photochemical processes of photosynthesis in plants. The study performed by these authors showed phytotoxic damage in *Pisum sativum*, affecting the content of photosynthetic pigments (chlorophyll *a*, *b* and carotenoids). In chlorophyll fluorescence parameters, the significant increase of $F0$ values and the decrease of F_V/F_M and Φ_{II} values was also recorded.

Our results demonstrated that the Fo value presented significant results for the 100 mg/L concentration (higher concentrations of nanoparticles have higher basal fluorescence). The difference between minimum fluorescence (F0) and maximum fluorescence (Fm) is the level of variable fluorescence (Fv) (Butler 1978; Genty et al 1992). The Fv / Fm ratio is a plant “health coefficient” (Butler 1978; Genty et al 1992). In leaves not exposed to stress, the Fv / Fm value is high (approximately 0.83) (Demmig and Björkman 1987). Our plants presented values of 0.8 for the Fv/Fm ratio, which allows us to conclude that they were healthy. For the light-adapted plants, we found that these had slightly higher values of minimum fluorescence, being higher in the concentration of 50 mg/L. Maximum fluorescence was also higher in this analysis, as was variable fluorescence. After the light stimulus, there is an initial increase in fluorescence. The fluorescence signal then decreases over a short period, which is

called "extinction" (Krause and Weis 1991). There is a set of processes that promote this extinction of the fluorescence signal. Initially there is a slight activation of the photosynthesis process, with the activation of essential enzymes in the Calvin cycle (Buchanan and Balmer 2005) as well as the increase in the size of groups of metabolites in the stroma and cytosol.

The opening of stomata increases the availability of CO_2 for RuBisCO. The stomata tend to open and close more slowly when photosynthetic events are not underway (Lawson et al 2012). These events allow the existence of a greater availability of receptors for the electrons derived from the processes dependent on the light in the thylakoid and contribute to the extinction by the process of photosynthesis itself. This removes excess excitation energy within chlorophyll-containing complexes avoiding the formation of harmful free radicals. This type of extinction competes with fluorescence and photochemical extinction, and acts as a "safe" mechanism to dissipate substantial levels of energy excitation of chlorophyll, depending on the conditions and species (Demmig-Adams and Adams 2006).

Photosynthesis presents a phase where the ATP and NADPH produced in the electron transport chain are used for the synthesis of carbohydrates. This phase of CO_2 fixation involves gas exchanges. The $TiSiO_4$ NPs had a negative effect on the intercellular CO_2 concentration. Relatively to the control, the 1 mg/L concentration of $TiSiO_4$ NPs exposure presented higher levels of stomatal conductance, photosynthetic and transpiration rate which is expected because a greater stomatal opening can be associated with a higher transpiration rate. These data support the possible decrease in the starch levels (trend to decrease in the 10 and 100 mg/L), which can be associated with a decrease in the Calvin cycle efficacy and / or metabolism associated with starch synthesis, or with an increase in starch consumption for plant growth.

The pigments content analysis shows that especially carotenoid content tends to decrease when exposed to $TiSiO_4$. Overall, these results indicate a decrease in the photosynthetic pigments in the chloroplasts. It is not yet known whether the NPs somehow increase their degradation or block their synthesis. A study in *Arabidopsis thaliana* showed TiO_2NPs exposure increased expression of genes associated with photosystem II (Ze et al 2011), which may explain the maintenance of the efficiency of this phase of photosynthesis, even with negative effects on the concentration of some pigments.

5. Conclusions and future perspectives

This work shows the effect of $TiSiO_4$ nanoparticles on plants, namely *L. sativa*, on germination, growth, oxidative stress, as well as possible cellular damages and photosynthetic processes.

To our knowledge this is the first study on these specific NPs impacts on the photosynthetic performance and some oxidative impacts on plants. We show that whilst germination is not greatly impaired, some effects on growth are observed, curiously mostly in the shoot, with an impairment of elongation paralleled with an increase in organs FW and DW.

Growth is highly dependent on the biomass and photosynthesis. Relatively to photosynthesis, conclusions were reinforced from previous studies for other substances (eg hydrocarbons), with changes in anthocyanin and chlorophyll levels. The efficiency of photosystem II (damage repair) was clarified indicating that plants adapted to dark had values often reported as healthy, even when exposed to the NPs/stress.

Our data also show that some variables are more sensitive to these NPs (biomass, chlorophyll content). Our results, indicate therefore that further studies should be performed with other species and/or other culture systems to evaluate if the most sensitive parameters can (and should) be used as sensitive markers in future studies.

Also, the apparent non-toxicity of lower doses of these NPs opens up prospects for: a) promising safety doses to be used in agro-industry and/or allowance of content in the environment; b) putative doses indicating their potential use in various economic sectors, but further studies have to be carried out.

Further studies regarding multiple aspects such as the impact of these NPs on crops' genomics, transcriptomics, proteomics and metabolomics would also be interesting to perform in the future, and would provide a crucial battery of information to develop a mechanistic profile of action of these NPs in the plant cell and impacts in plant's performance (eg. yield). Also, microscopy studies to analyze the NP translocation, quantification of NPs in the plants' organs, and / or studies in real soil scenarios to study bioactivity in field conditions, impacts on rhizosphere, as well as putative impacts and bioaccumulation in the food chain, are fields open to investigation to better understand how these (and other) TiNPs behave in the ecosystem and how they enter, accumulate, and interact with other molecules in the cell.

Finally, whilst *L. sativa* proved to be a good study model, providing profitable data, supporting previous studies in the group with other NPs (eg. Couto 2016), the

knowledge in these NPs phytotoxicity would benefit using other species such as tomato (eg. accumulation in the fruit).

6. References

Aitken, R. J., Chaudhry, M. Q., Boxall, A. B., & Hull, M. (2006). Manufacture and use of nanomaterials: Current status in the UK and global trends. Occupational Medicine, 56(5), 300-306.

Aliabadi, T., Afshar, A. S., & Nematpour, F. S. (2016). The effects of nano TiO_2 and Nano aluminium on the growth and some physiological parameters of the wheat (*Triticum aestivum*). Iranian Journal of Plant Physiology, 6(2).

Anbarasu, M., Anandan, M., Chinnasamy, E., Gopinath, V., & Balamurugan, K. (2015). Synthesis and characterization of polyethylene glycol (PEG) coated Fe_3O_4 nanoparticles by chemical co-precipitation method for biomedical applications. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 135, 536-539.

Anpo, M., Nakaya, H., Kodama, S., Kubokawa, Y., Domen, K., & Onishi, T. (1986). Photocatalysis over binary metal oxides. Enhancement of the photocatalytic activity of titanium dioxide in titanium-silicon oxides. The Journal of Physical Chemistry, 90(8), 1633-1636.

Bouguerra, S., Gavina, A., Ksibi, M., da Graça Rasteiro, M., Rocha-Santos, T., & Pereira, R. (2016). Ecotoxicity of titanium silicon oxide ($TiSiO_4$) nanomaterial for terrestrial plants and soil invertebrate species. Ecotoxicology and Environmental Safety, 129, 291-301.

Bradford, M.M. (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248–254

Buchanan, B.B., Balmer, Y. (2005). Redox regulation: a broadening horizon. Annual Review of Plant Biology 56, 187–220.

Butler W.L. (1978). Energy distribution in the photochemical apparatus of photosynthesis. Annual Review of Plant Physiology 29, 457–478.

Buzea, C., Pacheco, I. I., & Robbie, K. (2007). Nanomaterials and nanoparticles: Sources and toxicity. Biointerphases, 2(4).

Castiglione, M. R., Giorgetti, L., Geri, C., & Cremonini, R. (2011). The effects of nano-TiO_2 on seed germination, development and mitosis of root tip cells of *Vicia narbonensis L.* and *Zea mays L.* Journal of Nanoparticle Research, 13(6), 2443-2449.

Clément, L., Hurel, C., & Marmier, N. (2013). Toxicity of TiO_2 nanoparticles to cladocerans, algae, rotifers and plants – Effects of size and crystalline structure. Chemosphere, 90(3), 1083-1090.

Comotto, M., Casazza, A. A., Aliakbarian, B., Caratto, V., Ferretti, M., & Perego, P. (2014). Influence of TiO_2 Nanoparticles on Growth and Phenolic Compounds Production in Photosynthetic Microorganisms. The Scientific World Journal, 1-9.

Corredor, E., Testillano, P. S., Coronado, M. J., González-Melendi, P., Fernández-Pacheco, R., Marquina, C., & Risueño, M. C. (2009). Nanoparticle penetration and transport in living pumpkin plants: in situ subcellular identification. BMC Plant Biology, 9(1), 45.

Couto, M. (2016) "Impacto de diferentes formulações de nanoparticulas de TiO_2 na fotossíntese e stress oxidativo de *Lactuca sativa*" Internship report. Faculty of Sciences. University of Porto, Portugal (60)

Cox, A., Venkatachalam, P., Sahi, S., & Sharma, N. (2016). Silver and titanium dioxide nanoparticle toxicity in plants: A review of current research . Plant Physiology and Biochemistry, 107, 147-163.

Cushnie, T.P.T., Robertson, P.K.J., Officer, S., Pollard, P.M., Prabhu, R., McCullagh, C., Robertson, J.M.C. (2010). "Photobactericidal effects of TiO_2 thin films at low temperature". Journal of Photochemistry and Photobiology A: Chemistry. 216 (2-3): 290–294.

Demir, E., Kaya, N., & Kaya, B. (2014). Genotoxic effects of zinc oxide and titanium dioxide nanoparticles on root meristem cells of *Allium cepa* by comet assay. Turkish Journal of Biology, 38, 31-39.

Demmig, B. & Björkman, O., (1987). Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. Planta, 170(4), 489-504.

Demmig-Adams, B., Adams, I. (2006). Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. New Phytologist 172, 11–21.

Dias, M. C., Monteiro, C., Moutinho-Pereira, J., Correia, C., Gonçalves, B., & Santos, C. (2013). Cadmium toxicity affects photosynthesis and plant growth at different levels. Acta Physiologiae Plantarum, 35(4), 1281-1289.

Dias, M.C., Pinto, G., Santos, C. (2011) Acclimatization of micropropagated plantlets induces an antioxidative burst: a case study with *Ulmus minor* Mill. Photosynthetica 49:259–266

Ellis, E. D., Watkins, J., Tankersley, W., Phillips, J., & Girardi, D. (2010). Mortality Among Titanium Dioxide Workers at Three DuPont Plants. Journal of Occupational and Environmental Medicine, 52(3), 303-309.

Feizi, H., Moghaddam, P. R., Shahtahmassebi, N., & Fotovat, A. (2011). Impact of Bulk and Nanosized Titanium Dioxide (TiO_2) on Wheat Seed Germination and Seedling Growth. Biological Trace Element Research, 146(1), 101-106.

Frazier, T. P., Burklew, C. E., & Zhang, B. (2013). Titanium dioxide nanoparticles affect the growth and microRNA expression of tobacco (*Nicotiana tabacum*). Functional & Integrative Genomics, 14(1), 75-83.

Gao, J., Xu, G., Qian, H., Liu, P., Zhao, P., & Hu, Y. (2013). Effects of nano-TiO_2 on photosynthetic characteristics of *Ulmus elongata* seedlings. Environmental Pollution, 176, 63-70.

Genty, B., Goulas, Y., Dimon, B., Peltier, G., & Moya, I. (1992). Modulation of efficiency of primary conversion in leaves, mechanisms involved at PSII. Research in photosynthesis, 4, 603-610.

Ghosh, M., Bandyopadhyay, M., & Mukherjee, A. (2010). Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: plant and human lymphocytes. Chemosphere, 81(10), 1253-1262.

Gonzalez-Melendi, P., Fernandez-Pacheco, R., Coronado, M. J., Corredor, E., Testillano, P. S., Risueno, M. C., Perez-De-Luque, A. (2008). Nanoparticles as Smart Treatment-delivery Systems in Plants: Assessment of Different Techniques of Microscopy for their Visualization in Plant Tissues. Annals of Botany, 101(1), 187-195.

Guichard, Y., Schmit, J., Darne, C., Gate, L., Goutet, M., Rousset, D., Rastoix, O., Wrobel, R., Witschger, O., Martin, A., Fierro, V., Binet, S., (2012). Cytotoxicity and genotoxicity of nanosized and microsized titanium dioxide and iron oxide particles in syrian hamster embryo cells. Annals of Occupational Hygiene. 56, 631e644.

Hackenberg, S., Friehs, G., Kessler, M., Froelich, K., Ginzkey, C., Koehler, C., Kleinsasser, N. (2010). Nanosized titanium dioxide particles do not induce DNA damage in human peripheral blood lymphocytes. Environmental and Molecular Mutagenesis, 52(4), 264-268.

Hatami, M., Ghorbanpour, M., & Salehiarjomand, H. (2014). Nano-anatase TiO_2 modulates the germination behavior and seedling vigority of some commercially important medicinal and aromatic plants. Journal of Biological and Environmental Sciences, 8(22).

Hussain, S., Thomassen, L. C., Ferecatu, I., Borot, M. C., Andreau, K., Martens, J. A., Fleury J., Baeza-Squiban A., Marano F. & Boland, S. (2010). Carbon black and titanium dioxide nanoparticles elicit distinct apoptotic pathways in bronchial epithelial cells. Particle and Fibre Toxicology, 7(1), 10.

Iavicoli, I., Leso, V., & Bergamaschi, A. (2012). Toxicological Effects of Titanium Dioxide Nanoparticles: A Review of In Vivo Studies. Journal of Nanomaterials, 2012, 1-36.

Ilisz, I., Dombi, A., Mogyorósi, K., & Dékány, I. (2003). Photocatalytic water treatment with different TiO_2 nanoparticles and hydrophilic/hydrophobic layer silicate adsorbents. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 230(1), 89-97.

Irigoyen, J. J., Emerich, D. W., & Sanchez-Diaz, M. (1992). Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiol Plant Physiologia Plantarum*, 84*(1), 55-60.

Jadhav, S. B., Phugare, S. S., Patil, P. S., & Jadhav, J. P. (2011). Biochemical degradation pathway of textile dye Remazol red and subsequent toxicological evaluation by cytotoxicity, genotoxicity and oxidative stress studies. International Biodeterioration & Biodegradation, 65(6), 733-743.

Jośko, I., & Oleszczuk, P. (2013). Influence of soil type and environmental conditions on ZnO, TiO_2 and Ni nanoparticles phytotoxicity. Chemosphere, 92(1), 91-99.

Karunakaran, G., Suriyaprabha, R., Rajendran, V., & Kannan, N. (2016). Influence of ZrO_2, SiO_2, Al_2O_3 and TiO_2 nanoparticles on maize seed germination under different growth conditions. IET nanobiotechnology, 10(4), 171-177.

Kaur, S., Maurya, A. (2016) Assessment of stress end points in *Vigna radiata* seedlings exposed to pre-activated TiO_2 and $TiSiO_4$ nanoparticles under solar radiation. International Journal of Pharmacy and Pharmaceutical Sciences, 8 (10), pp. 198-203.

Klančnik, K., Drobne, D., Valant, J., & Koce, J. D. (2011). Use of a modified *Allium* test with nanoTiO_2. Ecotoxicology and Environmental Safety, 74(1), 85-92.

Koce, J. D., Drobne, D., Klančnik, K., Makovec, D., Novak, S., & Hočevar, M. (2014). Oxidative potential of ultraviolet-A irradiated or nonirradiated suspensions of titanium dioxide or silicon dioxide nanoparticles on *Allium cepa* roots. Environmental Toxicology and Chemistry, 33(4), 858-867.

Kondrakov, A. O., Ignatev, A. N., Frimmel, F. H., Bräse, S., Horn, H., & Revelsky, A. I. (2014). Formation of genotoxic quinones during bisphenol A degradation by TiO_2 photocatalysis and UV photolysis: a comparative study. Applied Catalysis B: Environmental, 160, 106-114.

Krause, G.H., Weis, E. (1991). Chlorophyll fluorescence and photosynthesis—the basics. Annual Review of Plant Physiology and Plant Molecular Biology 42, 313–349.

Kummerová, M., Krulová, J., Zezulka, Š., & Tříska, J. (2006). Evaluation of fluoranthene phytotoxicity in pea plants by Hill reaction and chlorophyll fluorescence. Chemosphere, 65(3), 489-496.

Kurepa, J., Paunesku, T., Vogt, S., Arora, H., Rabatic, B. M., Lu, J., & Smalle, J. A. (2010). Uptake and distribution of ultrasmall anatase TiO_2 Alizarin red S nanoconjugates in *Arabidopsis thaliana*. Nano letters, 10(7), 2296-2302.

Larue, C., Laurette, J., Herlin-Boime, N., Khodja, H., Fayard, B., Flank, A., Carriere, M. (2012). Accumulation, translocation and impact of TiO_2 nanoparticles in wheat (*Triticum aestivum* spp.): Influence of diameter and crystal phase. Science of The Total Environment, 431, 197-208.

Lawson, T., Oxborough, K., Morison, J. I., & Baker, N. R. (2002). Responses of photosynthetic electron transport in stomatal guard cells and mesophyll cells in intact leaves to light, CO_2, and humidity. Plant Physiology, 128(1), 52-62.

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. Journal of Experimental Botany, 51(345), 659-668.

Mohammadi, R., Maali-Amiri, R., & Abbasi, A. (2013). Effect of TiO_2 nanoparticles on chickpea response to cold stress. Biological Trace Element Research, 152(3), 403-410.

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum, 15(3), 473-497.

Murchie, E. H., & Horton, P. (2007). Toward C4 rice: learning from the acclimation of photosynthesis in the C3 leaf. Charting New Pathways to C4 Rice, 333-351.

Nel, A., Xia, T., Mädler, L. & Li, N. (2006). Toxic Potential of Materials at the Nanolevel. Science, 311(5761), 622-627.

OECD - Organization for Economic Cooperation and Development, 1984. Terrestrial Plants, GrowthTest. OECD – Guideline for Testing of Chemicals, Paris, France, p.208.

Osaki, M., Shinano, T., & Tadano, T. (1991). Redistribution of carbon and nitrogen compounds from the shoot to the harvesting organs during maturation in field crops. Soil Science and Plant Nutrition, 37(1), 117-128.

Pakrashi, S., Jain, N., Dalai, S., Jayakumar, J., Chandrasekaran, P.T., Raichur, A.M., Chandrasekaran, N., Mukherjee, A. (2014) In Vivo Genotoxicity Assessment of Titanium Dioxide Nanoparticles by *Allium cepa* Root Tip Assay at High Exposure Concentrations. PLoS One 9(2)

Peltier, G., & Cournac, L. (2002). Chlororespiration. Annual review of plant biology, 53(1), 523-550.

Piccinno, F., Gottschalk, F., Seeger, S., & Nowack, B. (2012). Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. Journal of Nanoparticle Research, 14(9).

Rafique, R., Arshad, M., Khokhar, M. F., Qazi, I. A., Hamza, A., Virk, N. (2014), Growth Response of Wheat to Titania Nanoparticles Application. NUST Journal of Engineering Sciences, 7(1) ,42-46

Raliya, R., Nair, R., Chavalmane, S., Wang, W., & Biswas, P. (2015). Mechanistic evaluation of translocation and physiological impact of titanium dioxide and zinc oxide nanoparticles on the tomato (*Solanum lycopersicum L*.) plant. Metallomics, 7(12), 1584-1594.

Ren, H. X., Liu, L., Liu, C., He, S. Y., Huang, J., Li, J. L. and Gu, N. (2011) Physiological investigation of magnetic iron oxide nanoparticles towards Chinese mung bean. Journal of Biomedical Nanotechnology, 7(5): 677-684.

Samadi, N. (2014). Effect of TiO_2 and TiO_2 Nanoparticle on Germination, Root and Shoot Length and Photosynthetic Pigments of *Mentha Piperita*. International Journal of Plant & Soil Science, 3(4), 408-418.

Satoh, N., Nakashima, T., & Yamamoto, K. (2013). Metastability of anatase: size dependent and irreversible anatase-rutile phase transition in atomic-level precise titania. Scientific reports, 3, 1959.

Shallan, A., Mm, H., Namich, A.A., Ibrahim, A.A. (2016). Biochemical and Physiological Effects of TiO_2 and SiO_2 Nanoparticles on Cotton Plant under Drought Stress. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 7. 1540-1551.

Shaw, A. K. and Z. Hossain. (2013). Impact of nanoCuO stress on rice (*Oryza sativa L*.) seedlings'. Chemosphere, 93(6): 906-915

Silva, S., Oliveira, H., Craveiro, S. C., Calado, A. J., & Santos, C. (2016). Pure anatase and rutile+ anatase nanoparticles differently affect wheat seedlings. Chemosphere, 151, 68-75.

Sims, D. A., & Gamon, J. A. (2002). Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sensing of Environment, 81(2), 337-354.

Song, U., Shin, M., Lee, G., Roh, J., Kim, Y., & Lee, E. J. (2013). Functional Analysis of TiO_2 Nanoparticle Toxicity in Three Plant Species. Biological Trace Element Research,155(1), 93-103

Strambeanu, N., Demetrovici, L., & Dragos, D. (2015). Anthropogenic Sources of Nanoparticles.Nanoparticles Promises and Risks, 21-54.

Tan, S. S.; L. Zou; E. Hu (2006). Photocatalytic reduction of carbon dioxide into gaseous hydrocarbon using TiO_2 pellets. Catalysis Today. 115: 269–273.)

Tumburu, L., Andersen, C. P., Rygiewicz, P. T., & Reichman, J. R. (2014). Phenotypic and genomic responses to titanium dioxide and cerium oxide nanoparticles in *Arabidopsis* germinants. Environmental Toxicology and Chemistry, 34(1), 70-83.

Wild, P., Bourgkard, E., & Paris, C. (2009). Lung Cancer and Exposure to Metals: The Epidemiological Evidence. Methods in Molecular Biology Cancer Epidemiology, 139-167.

Yang, Z., Chen, J., Dou, R., Gao, X., Mao, C., & Wang, L. (2015). Assessment of the Phytotoxicity of Metal Oxide Nanoparticles on Two Crop Plants, Maize (*Zea mays L.*) and Rice (*Oryza sativa L*.). International Journal of Environmental Research and Public Health, 12(12), 15100-15109.

Ze, Y., Liu, C., Wang, L., Hong, M., Hong, F. (2011). The regulation of TiO_2 nanoparticles on the expression of light-harvesting complex II and photosynthesis of chloroplasts of *Arabidopsis thaliana*. Biol Trace Elem Res. Nov;143(2):1131-41.

Zhang, S., Gao, H., & Bao, G. (2015). Physical Principles of Nanoparticle Cellular Endocytosis. ACS Nano, 9(9), 8655-8671.

Zheng, L., Hong, F., Lu, S., & Liu, C. (2005). Effect of Nano-TiO on Strength of Naturally Aged Seeds and Growth of Spinach. Biological Trace Element Research, 104(1), 083-092.